On page 10, please replace the second paragraph with the following:

FIG. 6 is a nucleotide sequence (SEQ ID No. 1) showing the DNA sequence of a region of the *E.coli* genome containing the sequence of the *dep* gene. This region of the *E. coli* genome is available at Accession No. AE000261 U00096. The sequence shown is that of nucleotides 4381-8280. The *dep* gene is encoded by nucleotides 4627-5838. The *dep* sequence is shown in brackets.

On page 10, please replace the third paragraph with the following:

FIG. 7 is a nucleotide sequence (SEQ ID No. 2) showing the isolated DNA sequence of the *dep* gene. The plasmid pSP007 was confirmed to contain the *dep* gene by obtaining DNA sequence data from the one end of the 1.7 kb insert. Sequence data obtained in this manner matched the first.

Please replace the paragraph bridging pages 12 and 13 with the following:

Using BLAST-homology search computer program, we carried out a homology search for the putative protein encoded by *dep*. Fig. 4 shows nine proteins showing significantly high homology with Dep. Half of these proteins confer resistance to chloramphenicol. The proteins showing the highest degree of homology include: Cmr (SEQ ID No. 4) from *Rhodococcus fascines* (Desomer *et al.*, 1992), CmrA (SEQ ID No. 5) from *R. erythropolis* (Nagy *et al.*, 1997), CmL (SEQ ID No. 6) from *Streptomyces lividans* 1326 (Dittrich *et al.*, 1991), Cmx (SEQ ID No. 7) from *Corynebacterium striatum* (Accession no U72639), and CmlV (SEQ ID No. 8) from *S. venezuelae* ISP5230 (Mosher *et al.*, 1995). As seen from Fig. 4, Dep (SEQ ID No. 3) has the highest degree of homology with Cmr(SEQ ID No. 4), product of chloramphenicol resistant gene (cmr) as compared to other proteins. Cmr protein was shown to contain three consensus sequences defined by Rouch *et al.* (1990) for transmembrane proteins. These sequences are at similar positions with respect to the predicted transmembrane domains. These are marked in Fig. 5 with dotted lines and are designated a I, II, III. In case of Dep, the first stretch (I) comprising of LP is completely homologous with the stretch defined by these authors. The second stretch (II) shows 50% similarity with that of Cmr protein and the third stretch (III) is homologous between these two proteins except for one residue. According to the model proposed by Rouch *et al.* (1990), the stretches I and III are located on the outside of the

cytoplasmic membrane and the stretch II is located on the inside of the membrane. The positions of the membrane loops for the putative protein encoded by qacA were ascertained by inspecting the antigenic index profile and turn prediction. Such regions have a high antigenic index and turn probability (Rouch *et al.,* 1990).

Please replace the second full paragraph on page 13 with the following:

The other proteins homologous to Dep include BcR (SEQ ID No. 9) (bicyclomycin-resistance protein) from *E. coli* (Bentley *et al.*, 1993), Bmr3 (SEQ ID No. 10) from *B. subtilis* involved in the multiple drug efflux pump conferring resistance to puromycin, tosufloxacin, norfloxacin (Ohki and Murata, 1997), Tet from *Staphlococcus hyicus* conferring tetracycline resistance (Schwarz *et al*, 1992) and YjcC conferring tetracenomycin-resistance (Accession no. D90826) (Fig. 4). All of these are efflux proteins, which is one of the most common mechanisms for drug resistance. We speculate that *dep* encodes a putative efflux protein that forms a cytoplasmic channel specific for DHCP. The homologies are more prominent towards the N-terminal end of the proteins, which also is a common feature for efflux proteins (Desomer et al., 1992)

Marked-Up Version Showing Changes Made to the Claims

7. (Amended) ~~A~~ An isolated and purified gene encoding *dep*, the DHCP efflux protein, wherein said DHCP efflux protein comprises the amino acid sequence (SEQ ID No. 3).

8. (Amended) The *dep* gene of Claim 7, wherein the *dep* gene is from *E. coli*, and wherein said *dep* gene confers resistance to DHCP or a functionally equivalent compound when present in multiple copies in a bacterial cell.

10. (Amended) A plasmid comprising the ~~*dep* gene~~ gene encoding the DHCP efflux protein which comprises the amino acid sequence of (SEQ ID No. 3), which plasmid confers expression of multiple copies of the ~~*dep* gene~~said gene in bacteria cells that have been transformed with said plasmid.

12. (Amended) Bacteria cells containing ~~multiple copies of~~ the plasmid of Claim 10, which are resistant to DHCP.

Kindly cancel Claims 1-6, 9, 11 and 13-14.

Marked-Up Version Showing Changes Made to the Abstract

The invention relates to a gene, *dep*, which confers resistance to the antibacterial activity of 4,5-dihydroxy-2-cyclopenten-1-one (DHCP). The invention further relates to the putatitve protein encoded by *dep*, derived from *Escherichia coli*, which is a hydrophobic transmembrane efflux protein specific for DHCP. The invention further relates to plasmids containing the *dep* gene, and to bacterial cells expressing *dep*. Furthermore, the invention provides applications for use in conferring resistance to antibacterial activity in organisms. The *dep* gene can be used to identify compounds which inhibit the efflux activity responsible for the resistance to DHCP or to compounds which are functionally equivalent to DHCP.

Remarks

The Applicants note with appreciation the Examiner's helpful comments and suggestions throughout the Office Action. The Applicants have amended the claims in accordance with many of the Examiner's suggestions.

Turning to the specifics of the Action, Applicants have amended the specification to identify the sequences with their appropriate SEQ ID No. Specifically, Applicants have amended Fig. 6 to identify the DNA sequence with SEQ ID No. 1, and amended Fig. 7 to identify the DNA sequence with SEQ ID No. 2. Concerning the need to amend Fig. 4, Applicants submit herewith a substitute copy of the sequence listing in a computer-readable form (CRF) and paper copy, along with an amendment requesting its entry into the specification, and a statement that the content of the paper and CRF copies are the same. Further, Applicants submit that as a result of this substitute copy of the sequence listing, no new matter has been added.

Turning now to the Examiner's objections to the specification, Applicants submit that as a result of the aforementioned amendments to Figs. 6 and 7, the objection is now obviated. Applicants note with appreciation the Examiner's modification regarding appropriate pagination as was discussed with the Applicants' representatives on October 23, 2002.

The Applicants have amended the Abstract in accordance with the Examiner's helpful advice, to include the full name of the source species, *Escherichia coli*. Further, the Applicants have amended the title in accordance with the Examiner's helpful suggestion so that the title now reads: "Gene encoding a 4,5-dihydroxy-2-cyclopenten-1-one (DHCP), efflux protein promoting resistance to DHCP."

Claims 9, 11, and 13 have been objected to under 37 CFR §1.75(c) as being of improper dependent form for failing to further limit the subject matter of a previous claim. Claims 9, 11 and 13 have been cancelled. Claims 1-6 and 14 have also been cancelled as being drawn to non-elected subject matter. The Applicants reserve the right to file one or more divisional applications directed to the subject matter thereof..

§112 Rejections

Claims 7-13 have been rejected under 35 U.S.C. §112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. The Examiner's helpfully suggest that, the Applicants change "DHCP" to "4,5-dihydroxy-2-cyclopenten-1-one (DHCP)." However, Claim 1 has been cancelled as set forth above.

Turning to the rejections under 35 U.S.C. §112, first paragraph, Applicants have amended independent Claims 7 and 10 to recite the structural characteristics of the *dep* gene. Applicants submit that the hydrophobic nature of the transmembrane protein encoded for by the *dep* gene, along with the knowledge of the *E. coli* genome, one skilled in the art would be able to predict the structure of the other members of this transmembrane protein. In light of the unique nature of the protein encoded for by the *dep* gene, along with the amendments to Claims 7, the Applicants submit that the genes, plasmids, and bacteria containing the genus of the *dep* gene are adequately described.

Claims 7 and 9-13 are rejected under 35 U.S.C. §112, first paragraph as lacking enablement. Applicants submit that as a result of amendments to Claim 7, one skilled in the art could make or use the invention commensurate within the scope of the claims. Applicants submit that the known genome of *E. coli* and the relative predictability of homologous sequences allow one skilled in the art to find the *dep* gene, or a structural and functional equivalent, from a variety of sources. Applicants further submit that given the particular nature of DHCP along with the knowledge of the *dep* gene, which Applicants have disclosed, one skilled in the art would be able to find a number of related and functional transmembrane proteins that could serve as an efflux pump to promote resistance to DHCP.

§101 Rejection

claim 7 has been amended to recite an "isolated and purified" gene in acordance with the Examiner's helpful suggestion. Withdrawal of the §101 rejection is respectfully requested.

§102 Rejections

Claims 7-9 have been rejected under 35 U.S.C. §102(b) as being anticipated by Blattner et al. (The Complete Genome Sequence of *Echerischia coli* K12. Science (1997) 277:1453-1474) and Gene Bank Accession No. AE000261. Applicants agree that Blattner et al. teaches a stretch of DNA from the *E. coli* genome, which necessarily encodes for the gene of Applicants' invention. However, Applicants have amended Claim 7 to reflect that the *dep* gene was isolated and purified. Applicant submits that Blattner et al. does not characterize the Applicants' isolated *dep* gene. There is no teaching in Blattner et al. that the *dep* gene sequence disclosed by the reference has the property of encoding for a protein that confers DHCP resistance. The Examiner is kindly asked to consider page 1458 of Blattner et al. wherein the reference makes broad classifications regarding *E. coli* gene products. Specifically, Blattner et al. classifies 281 transport proteins in terms of broad functions relating to a general ability transport or bind to cellular material, while leaving 1632 proteins unclassified. Blattner et al.does not distinguish the *dep* gene from any of the multitude of other genes encoding for transport proteins, nor does the reference classify the sequence encoding the *dep* gene as a transport protein encoding gene. In light of the broad study of the *E. coli* genome presented by Blattner et al. it is clear that the reference fails to show an isolated and purified *dep* gene functional to confer DHCP resistance and as a result, Blattner et al. fails to anticipate the Applicants' claims as amended.

§103 Rejections

Claims 10-13 have been rejected under 35 U.S.C. §103(a) as being unpatentable over Blattner et al. and Gene Bank Accession No. AE000261 in view of Weichert et al. (Optimization of Heterologous Protein Production in *Escherichia coli*. (Current Opinion in Biotechnology (1996)

7:494-499). The rejection urges that while Blattner et al. fails to teach a gene in a multi-copy plasmid, the teachings of Weichert et al. disclose heterologous expression of proteins in *E. coli* using multi-copy plasmids, and thus it would have been obvious to one of ordinary skill in the art to combine the aforementioned references. Applicants respectfully traverse this assertion. Applicants submit that as a result of the amendments and in light of the arguments set forth below, this rejection is now obviated. Applicants respectfully submit that there is no motivation found in the prior art whereby a person of ordinary skill in the field of this invention would have motivation to insert the isolated gene taught by Applicants into a multi-copy plasmid.

When considering the appropriate test of obviousness under 35 U.S.C. §103 it must be kept in mind that

> Where claimed subject matter has been rejected in view of a combination of prior art references, a proper analysis under <103 requires, *inter alia* consideration of two factors: (1) whether the prior art would have suggested to those of ordinary skill in the art that they should make the claim composition or device, or carry out the claim process; and (2) whether the prior art would also have revealed in so making or carrying out, those of ordinary skill would have a reasonable expectation of success. See *In re Dow Chemical Co.*, 435 USPQ 2d 1529, 1531 (Fed. Cir. 1988). Both the suggestion and the reasonable expectation of success must be found in the prior art, not in the Applicants' disclosure. *Id. In re Vaeck*, 20 USPQ 2d 1438, 1442 (Fed. Cir. 1991).

Weichert et al. is merely a review of the variety of methods that have been used to optimize protein production in *E. coli*. Weichert et al states:

> The production of active/functional proteins is frequently quite challenging. This review will update advances in promoter options and induction techniques, and describe a variety of approaches that may be generally useful for improving the expression of functional proteins.

Nothing in Weichert et al. suggests that the use of multi-copy plasmids would achieve good production for the highly hydrophobic transport protein produced by the *dep* gene, or that high

expression of the Applicants' transport protein would result in the transmembrane protein serving as an efflux pump for DHCP.

The Examiner's frank acknowledgement on page 9 of the Official Action states that "very little is known about the antibacterial DHCP and about any transmembrane proteins that can serve as efflux pumps to promote resistance to DHCP." Given this admittedlack of knowledge, along with the highly hydrophobic nature of the transmembrane proteins encoded for by the *dep* gene, one of ordinary skill would not predict the successful integration and resulting hyper-expression of the *dep* gene via a multi-copy plasmid. Weichert et al. illustrates that gene doses can be manipulated by a plasmid copy number, not that all gene dosage will be manipulated by plasmid copy number. Weichert et al. merely offers manipulation of plasmid copy number, along with a litany of other gene dosage methods, as one potential to increase protein expression.

Furthermore, there is no reasonable expectation of success of achieving high gene dosage through multi-copy plasmids because plasmid loss can increase tremendously in the case of a very high copy number of plasmids, especially if the plasmid-borne genes are toxic to the hosts or otherwise significantly effect the plasmids growth rate. In light of this understanding, one skilled in the art could not predict the effect of the effect of hyper-expression of the *dep* gene on the host plasmid. It would be sperulative at best. A large amount of *dep* gene could have resulted in a concomitant accumulation of the desired protein, which would require a high level of an mRNA which, in turn, can cause destruction and cell death resulting in expression instability. The hyper-expression of the *dep* genes transmembrane proteins which may have led to membrane lisis.

In view of the difficulties associated with hyper-expression of gene products in using multi-copy plasmids, and further in view of the prior art failing to suggest and provide a reasonable expectation of success of the expression of *dep* gene products for the transport of DHCP, Applicants respectfully submit that the rejection is now obviated.

Until Applicants' discovery, nothing in the prior art suggested that the hyper-expression of the *dep* gene products would function to transport DHCP.

In light of the foregoing, Applicants submit that the entire Application is now in complete condition for allowance, which is respectfully requested.

Respectfully submitted,

T. Daniel Christenbury
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Attorney for Applicants

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In the Specification (clean copy as amended)

Please replace the Title with the following:

A10

Gene encoding a 4,5-dihydroxy-2-cyclopenten-1-one (DHCP), efflux protein promoting resistance to DHCP

On page 9, please replace the last paragraph with the following:

A1

FIG. 4 The sequence homology between DEP(SEQ ID No. 3), Cmr (SEQ ID No. 4), CmrA (SEQ ID No. 5), CMl (SEQ ID No. 6), Cmx (SEQ ID No. 7), CmlV (SEQ ID No. 8), BcR (SEQ ID No. 9), Bmr3 (SEQ ID No. 10), YjcC (SEQ ID No. 11) and Tet (SEQ ID No. 12). Identical and similar sequences are marked with black and gray boxes, respectively. The consensus sequences for transmembrane proteins are marked with dotted lines and are represented as I, II, and III stretches.

On page 10, please replace the second paragraph with the following:

A2

FIG. 6 is a nucleotide sequence (SEQ ID No. 1) showing the DNA sequence of a region of the *E.coli* genome containing the sequence of the *dep* gene. This region of the *E. coli* genome is available at Accession No. AE000261 U00096. The sequence shown is that of nucleotides 4381-8280. The *dep* gene is encoded by nucleotides 4627-5838. The *dep* sequence is shown in brackets.

On page 10, please replace the third paragraph with the following:

A3

FIG. 7 is a nucleotide sequence (SEQ ID No. 2) showing the isolated DNA sequence of the *dep* gene. The plasmid pSP007 was confirmed to contain the *dep* gene by obtaining DNA sequence data from the one end of the 1.7 kb insert. Sequence data obtained in this manner matched the first.

Please replace the paragraph bridging pages 12 and 13 with the following:

A4

Using BLAST-homology search computer program, we carried out a homology search for the putative protein encoded by *dep*. Fig. 4 shows nine proteins showing significantly high homology with Dep. Half of these proteins confer resistance to chloramphenicol. The proteins showing the highest degree of homology include: Cmr (SEQ ID No. 4) from *Rhodococcus fascines* (Desomer *et al.*, 1992), CmrA (SEQ ID No. 5) from *R. erythropolis* (Nagy *et al.*, 1997), CmL (SEQ ID No. 6) from *Streptomyces lividans* 1326 (Dittrich *et al.*, 1991), Cmx (SEQ ID No. 7) from *Corynebacterium striatum* (Accession no U72639), and CmlV (SEQ ID No. 8) from *S. venezuelae* ISP5230 (Mosher

et al., 1995). As seen from Fig. 4, Dep (SEQ ID No. 3) has the highest degree of homology with Cmr(SEQ ID No. 4), product of chloramphenicol resistant gene (cmr) as compared to other proteins. Cmr protein was shown to contain three consensus sequences defined by Rouch *et al.* (1990) for transmembrane proteins. These sequences are at similar positions with respect to the predicted transmembrane domains. These are marked in Fig. 5 with dotted lines and are designated a I, II, III. In case of Dep, the first stretch (I) comprising of LP is completely homologous with the stretch defined by these authors. The second stretch (II) shows 50% similarity with that of Cmr protein and the third stretch (III) is homologous between these two proteins except for one residue. According to the model proposed by Rouch *et al.* (1990), the stretches I and III are located on the outside of the cytoplasmic membrane and the stretch II is located on the inside of the membrane. The positions of the membrane loops for the putative protein encoded by qacA were ascertained by inspecting the antigenic index profile and turn prediction. Such regions have a high antigenic index and turn probability (Rouch *et al.,* 1990).

Please replace the second full paragraph on page 13 with the following:

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In the Claims (clean copy as amended)

sub B1

7. (Amended) An isolated and purified gene encoding *dep*, the DHCP efflux protein, wherein said DHCP efflux protein comprises the amino acid sequence (SEQ ID No. 3).

A6

8. (Amended) The *dep* gene of Claim 7, wherein the *dep* gene is from *E. coli,* and wherein said *dep* gene confers resistance to DHCP or a functionally equivalent compound when present in multiple copies in a bacterial cell.

A7

10. (Amended) A plasmid comprising the gene encoding the DHCP efflux protein which comprises the amino acid sequence of (SEQ ID No. 3), which plasmid confers expression of multiple copies of the said gene in bacteria cells that have been transformed with said plasmid.

A8

12. (Amended) Bacteria cells containing the plasmid of Claim 10, which are resistant to DHCP.

B2 added 7

In the Abstract (clean copy as amended)

The invention relates to a gene, *dep*, which confers resistance to the antibacterial activity of 4,5-dihydroxy-2-cyclopenten-1-one (DHCP). The invention further relates to the putatitve protein encoded by *dep*, derived from *Escherichia coli,* which is a hydrophobic transmembrane efflux protein specific for DHCP. The invention further relates to plasmids containing the *dep* gene, and to bacterial cells expressing *dep*. Furthermore, the invention provides applications for use in conferring resistance to antibacterial activity in organisms. The *dep* gene can be used to identify compounds which inhibit the efflux activity responsible for the resistance to DHCP or to compounds which are functionally equivalent to DHCP.

A9